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Comparison of Five Routine Methods with the Candidate Reference Method for the Determination of Bilirubin in Neonatal Serum

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Summary: Using five routine methods and the candidate reference method of *Doumas* (Clin. Chem. *31*, 1779–1789 (1985)), total bilirubin was determined in 77 neonatal serum samples (concentration range 63–444 μmol/l, average value 227 μmol/l).

Four of the routine methods (*Jendrassik & Grof*'s (Biochem. Z. *297*, 81–89 (1938)) method, *Hertz*'s (Scand. J. Clin. Lab. Invest. *33*, 215–230 (1974)) method, the bilirubinometer procedure, and the method employing 2,5-dichlorophenyldiazonium (Scand. J. Clin. Lab. Invest. *29*, Suppl. 126, Abstr. 11. 12. (1972))) gave values that were generally higher than those of the reference method. In contrast, the results from *Vink*'s (Clin. Chem. *34*, 67–70 (1988)) direct spectrophotometric method differed only negligibly from those of the reference method. The accuracy of *Jendrassik & Grof*'s method, and to a limited extent that of the 2,5-dichlorophenyldiazonium method, can be improved by redetermination of the molar absorption coefficient, or by using a standard containing a matrix of human albumin, with an assigned value determined by the reference method. It was found that *Hertz*'s direct spectrophotometric method can be replaced by that of *Vink*.

The accuracy of the bilirubinometer results could be improved only by using calibrators with assigned values specific for the bilimeter, or by calibration with a serum pool.

Introduction

Owing to its great diagnostic importance, the bilirubin concentration of neonatal serum must be determined with high precision and accuracy (1).

Collaborative interlaboratory surveys, however, have revealed an unsatisfactorily wide scatter of results for the determination of bilirubin under similar operational conditions, and systematic differences between the results from the various routine methods of determination (2, 3). It is not yet known whether and to what extent these differences also occur in the analysis of native neonatal sera, or whether the problem is due to the matrices of the control sera.

In 1985, *Doumas* et al. (4) described a method for the determination of total bilirubin, which largely meets the requirements of a reference method. This was used as the "reference method" in the present study.

In addition, lyophilized bilirubin samples have recently become available, which contain a matrix of human albumin, and which have assigned values established by weighing the analyte and by determination with the reference method (5, 6). According to *Vink* et al. (5), it may be possible to use this material as a universal calibrator for the determination of bilirubin.

In the present work, the results from five routine methods and a micro-version of the reference method were compared, and their performance was evaluated by analysis of neonatal samples and standards.

Materials and Methods

Native samples

Seventy-seven samples of neonatal venous blood were analysed by 6 different methods. In addition, 12 serum samples were analysed by only the candidate reference method and the 2,5-dichlorophenyldiazonium method.

To obtain enough material for analysis, it was sometimes necessary to pool the residues from 2 different sera with similar bilirubin concentrations. Visibly haemolytic samples (i. e. haemoglobin > 1 g/l) were excluded.

Until analysis, serum samples were stored at −20 °C in aliquots of 500 µl; after thawing, they were centrifuged, then analysed within 6 hours.

All operations were conducted without laboratory illumination and in subdued light.

Control sera

Three control samples were analysed in each series. Two samples were obtained from the Rijksinstituut voor Volksgezondheid en Milieuhygiene, Bilthoven, The Netherlands; these were lyophilized solutions of bilirubin and human albumin (5, 6): P1 (Lot-No. 870624) certified value 308.1 µmol/l, and P2 (Lot-No. 870622) certified value 106.1 µmol/l. The third sample was "Sta-bil" (manufactured by Baxter, Lot-No. BIC 975), which also contains human albumin.

In order to determine precision in series, and to investigate the agreement between the reference method (4) and our micro-version, we analysed both "Sta-bil" and the control serum "Precibil" (manufactured by Boehringer Mannheim, Lot-No. 157908; containing human serum as the matrix).

Photometer

For methods 1 – 5, all measurement were performed in a double beam spectrophotometer (Perkin Elmer 554) with a band width of 1 nm, using quartz cuvettes (Suprasil, manufactured by Hellma, light path 10 mm).

The accuracy of the wavelength setting was checked with holmium nitrate solution (λ_{max} = 536.7 nm and 640.0 nm) and the emission line of the deuterium lamp (λ = 656.1 nm). Errors of less than 0.5 nm were found.

Absorption accuracy was monitored with haemiglobin cyanide solutions and gray filters. The assigned values for the gray filters (λ = 598 nm) and haemiglobin cyanide solutions (λ = 546.1 nm) were determined by the Physikalisch-Technische Bundesanstalt, Berlin.

For direct spectrophotometry without dilution, we used a bilirubinometer ("Bilimeter" from Ortho Diagnostic Systems GmbH) with the capillaries recommended by the manufacturer. According to the manufacturer, the bilimeter measures light absorption at 455 nm and 575 nm. For calibration purposes, we used "Sta-bil" (Lot BIC 975, with a value of 332 µmol/l quoted for use with bilimeters).

Pipetting, dilution and mixing

In addition to officially calibrated glass pipettes (from Brand, Wertheim), we also used a Microlab-M-Dilutor (manufactured by Hamilton) and a Multipette 4780 (manufactured by Eppendorf-Gerätebau). Precision and accuracy of the apparatus were tested according to the recommendations of the National Committee for Clinical Laboratory Standards USA (NCCLS Vol 4, No. 6). A vortex mixer was used for mixing.

Measurement technique

All measurements were made against air, and the position of the cuvette in the cuvette holder was unchanged during the entire series. Sample blanks and analytical samples were measured after each other in series. Before each spectrophotometric measurement the cuvette was washed at least once with the solution under test. After each measurement, the contents of the cuvette were sucked out with a pump.

After every ten measurements, the cuvette was filled with reagent solution to test for photometer drift (for details see l. c. (4)).

Analytical methods

1. "Reference method" (4)

Reagents

Caffeine solution:	
Sodium acetate	0.68 mol/l
Sodium benzoate	0.39 mol/l
EDTA	3.0 mmol/l
Caffeine	0.193 mol/l
Tartrate solution:	
Potassium sodium tartrate	1.13 mol/l
NaOH	1.88 mol/l
Sulphanilic acid solution:	
Sulphanilic acid	0.029 mol/l
HCl	0.47 mol/l
Sodium nitrite solution:	
Sodium nitrite	0.072 mol/l

Diazo-reagent:
Sulphanilic acid solution (10 ml) mixed with sodium nitrite solution (0.25 ml)

Procedure (micro-version): Serum (50 µl) was mixed with caffeine reagent (400 µl). After 10 min, diazo-reagent (100 µl) was added. The mixture was allowed to stand for 10 min, followed by the addition of tartrate solution (300 µl). The mixture was allowed to stand for a further 10 min before determination of absorption. The sample blank was prepared in the same way as the sample, except that sulphanilic acid solution (100 µl) was added instead of diazo-reagent. Measurements were performed at 598 nm against air (for details see l. c. (4)).

Calculation: c (µmol/l) = $(A_P - A_L) \times 225.2$.

In this micro-version all volumes are 10 times less than those used in the original version; the procedures for performance of the assay are unchanged.

2. Jendrassik & Grof's method (7)

Reagents were obtained from Boehringer Mannheim (order No. 123927).

Reagents

Caffeine solution:	
Sodium benzoate	0.52 mol/l
Caffeine	0.26 mol/l
Tartrate solution:	
Potassium sodium tartrate	0.93 mol/l
NaOH	1.9 mol/l
Sulphanilic acid solution:	
Sulphanilic acid	0.029 mol/l
HCl	0.17 mol/l
Sodium nitrite solution:	
Sodium nitrite	0.025 mol/l

Procedure: Sulphanilic acid solution (100 µl) and sodium nitrite solution (20 µl) (replaced by 20 µl distilled water in the sample blank) were pipetted into an Eppendorf reaction vessel, then mixed with caffeine solution (400 µl) and serum (50 µl). After 15 min, tartrate solution (400 µl) was added and mixed, and the absorption was measured at 578 nm after 15 min.

Calculation: c (µmol/l) = $A_P - A_L) \times 293$.

3. 2,5-Dichlorophenyldiazonium (DPD) method (8)

Reagents were obtained from Boehringer Mannheim (order No. 124943).

Reagents

Diazo-reagent:	
2,5-Dichlorophenyldiazonium chloride	1 mmol/l
HCl	0.1 mol/l
Detergent	
Blank value reagent:	
HCl	0.1 mol/l
Detergent	

Procedure: Serum (20 µl) was mixed with diazo-reagent (1000 µl) in an Eppendorf reaction vessel, and absorbance measured at 546 nm after 15 min. For the sample blank, diazo-reagent was replaced by blank value reagent (1000 µl).

Calculation: c (µmol/l) = $(A_P - A_L) \times 1112$.

4. Direct spectrophotometry according to Hertz et al. (9)

Procedure: Serum (50 µl) was diluted with 1000 µl of potassium tetraborate buffer (100 mmol/l, pH 9.3), and absorption was measured immediately at 466 nm and 522 nm.

Calculation: c (µmol/l) = $21 \times (21.6 \times A_{466} - 27.4 \times A_{522})$.

5. Direct spectrophotometry according to Vink (10)

Caffeine solution:	
Sodium acetate	1.52 mol/l
Sodium benzoate	0.52 mol/l
EDTA	3 mmol/l
Caffeine	0.257 mol/l

Procedure: Serum (50 µl) was diluted with caffeine reagent (1000 µl). Absorption of the mixture was measured within 15 min at 465 nm and 528 nm.

Calculation: c (µmol/l) = $21 \times 21.6 (A_{465} - A_{528})$.

6. Direct spectrophotometry without dilution (11)

Serum samples were added to capillary cuvettes and absorption was measured in the bilimeter according to the procedure of the manufacturer. The zero of the instrument was adjusted before each measurement. The instrument was calibrated with "Sta-bil" Lot 975, using the assigned value of the manufacturer. Results are given directly in µmol/l in a digital read-out.

Transformation of the analytical results with the aid of a standard

The analytical results (A_i) were multiplied by the quotient derived by dividing the assigned value of P1 (308.1 µmol/l) by the analytical result of the control sample P1 in the respective series (A_{P1})

$$A_t = A_i \times \frac{308.1}{A_{P1}} \text{ (µmol/l)}.$$

Statistics

For the comparison of methods the regression line was calculated by the method of *Passing & Bablok* (12).

Results and Discussion

Precision in series

Data for precision in series (N = 20) are shown in table 1 for the control samples "Sta-bil" and "Precibil". The greatest imprecision was shown by the DPD method with a variation coefficient of 1.1%, whereas the other procedures showed markedly smaller scatters. For this reason, duplicate values were not considered necessary for the method comparison.

Tab. 1. Analytical results and precision in series (N = 20) for the control sera "Sta-bil" and "Precibil" determined by 7 different methods ($\bar{x}$ = average value, CV = coefficient of variation).

No.	Method	Sta-bil		Precibil	
		$\bar{x}$ (µmol/l)	CV (%)	$\bar{x}$ (µmol/l)	CV (%)
1a	*Doumas* (original version)	318.7	0.5	269.6	0.5
1b	*Doumas* (micro version)	319.5	0.3	270.1	0.5
2	*Jendrassik & Grof*	314.1	0.4	277.1	0.3
3	DPD	324.1	1.1	292.4	1.1
4	*Hertz*	329.6	0.8	—	—
5	*Vink*	320.4	0.3	—	—
6	Bilimeter	332.9	1.2	313.8	0.5

Comparison of results from the candidate reference method of *Doumas* (4) with those from our micro-version of the method showed no significant differences between the scatter of values and the average values of the two control samples (which contain human albumin or human serum as the matrix). In the subsequent work, therefore, only the micro-version was used.

Precision from day to day and accuracy in control samples

Results for control samples P1, P2 and "Sta-bil" are shown in table 2. Two of the routine methods are based on the measurement of a coloured azo-pigment, and the scatter of values for these methods was markedly higher than for *Doumas*' and the other methods, which use direct spectrophotometry. Satisfactory agreement with the certified value of P1 was achieved only by methods 1 and 5 (methods of *Doumas* and *Vink*), with deviations of 0.32% and 0.75%, respectively. Variations in this value were also found by *Vink* et al. (10). In the analysis of P2, these same two methods gave values that differed from the certified value by −1.1% and 0.75%, respectively. Method 2 also produced values for P2 that differed only slightly (1.4%) from the certified value.

Recorded values for the control sample "Sta-bil" also depended markedly on the method of determination. The results of these analyses, however, also appeared to be influenced by the matrix of the control material. This becomes very apparent if the results are calculated using sample P1 (which has the same matrix as P2) as the standard (tab. 3). The values for P2 from the various methods then show smaller differences. The bilimeter results were an exception, still showing an appreciable deviation from the other analyses, but this can be attributed to the non-linearity of the instrument response in this analytical range (13). On the other hand, in the analysis of "Sta-bil", the differences were amplified when the calculation was based on standardization with P1.

Standardization with P1 also improved the precision from day to day for the analysis of P2. For the analysis of "Sta-bil", however, some methods even showed a greater scatter when the calculation was based on standardization with P1.

Comparison of methods

The results of the method comparison are shown in fig. 1a−1e as "residual plots". Since the linear measurement region of the bilirubinometer does not extend

Tab. 2. Analytical results and precision from day to day (N = 18) for control sera P1 (assigned value 308.1 µmol/l), P2 (assigned value 106.1 µmol/l) and "Sta-bil" (assigned values: *Jendrassik & Grof* 318 µmol/l, DPD 313 µmol/l, bilimeter 332 µmol/l) determined by 6 different methods ($\bar{x}$ = average value, CV = coefficient of variation).

No.	Method	P1		P2		Sta-bil	
		$\bar{x}$ (µmol/l)	CV (%)	$\bar{x}$ (µmol/l)	CV (%)	$\bar{x}$ (µmol/l)	CV (%)
1b	*Doumas* (micro version)	307.1	1.1	104.9	1.3	316.4	1.3
2	*Jendrassik & Grof*	317.3	1.7	108.0	3.5	312.6	2.1
3	DPD	329.8	2.5	114.2	2.4	322.9	2.5
4	*Hertz*	303.2	1.0	102.3	2.0	334.1	1.1
5	*Vink*	305.8	1.1	105.3	1.3	317.6	0.8
6	Bilimeter	298.9	1.1	114.3	2.0	332.6	1.2

Tab. 3. Analytical results and precision from day to day (N = 18) for control sera P2 and "Sta-bil". The results were calculated using control serum P1 (certified value 308.1 µmol/l) as the standard.

No.	Method	P2		Sta-bil	
		$\bar{x}$ (µmol/l)	CV (%)	$\bar{x}$ (µmol/l)	CV (%)
1b	*Doumas* (micro version)	105.3	1.1	317.4	1.8
2	*Jendrassik & Grof*	104.9	1.5	303.6	2.6
3	DPD	106.7	2.0	302.3	1.8
4	*Hertz*	104.0	1.7	339.5	1.0
5	*Vink*	106.1	1.5	320.0	1.4
6	Bilimeter	117.8	2.0	342.8	2.0

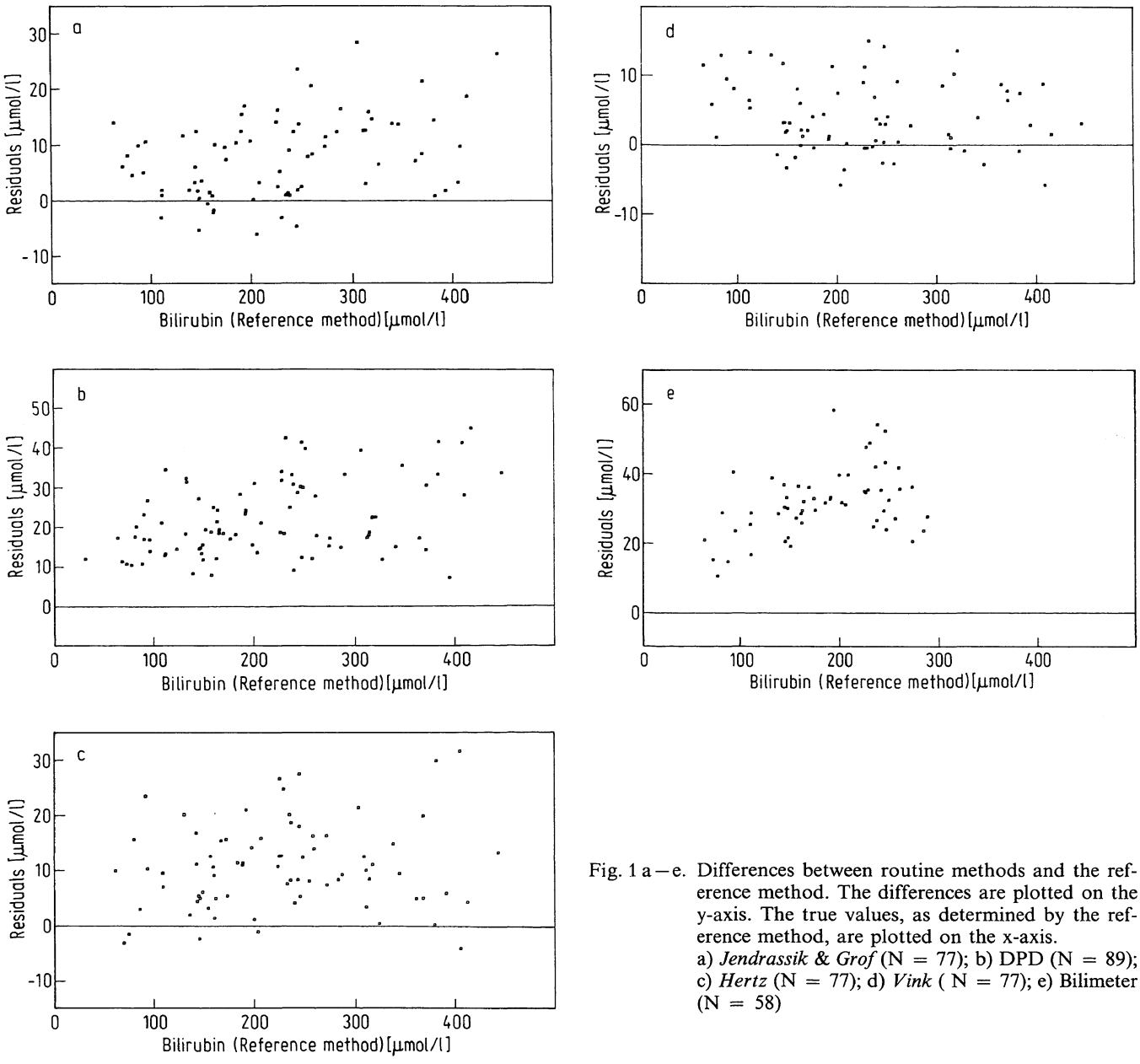

Fig. 1 a–e. Differences between routine methods and the reference method. The differences are plotted on the y-axis. The true values, as determined by the reference method, are plotted on the x-axis.
a) *Jendrassik & Grof* (N = 77); b) DPD (N = 89); c) *Hertz* (N = 77); d) *Vink* (N = 77); e) Bilimeter (N = 58)

beyond 300 μmol/l (13), sera with higher concentrations were omitted from the method comparison. Statistical data of the regression analysis are summarized in table 4.

All the routine methods gave values that were generally higher than those from the reference method. The smallest differences were found for *Vink*'s direct spectrophotometric method (fig. 1d), while the results from the bilirubinometer showed the greatest differences (fig. 1e). These two methods also showed respectively the best (r = 0.999) and the worst (r = 0.991) correlation with the reference method.

There are several reasons for the differences between the results from the candidate reference method and the routine method of *Jendrassik & Grof*, which is based on the same principle. As early as 1973, *Doumas* et al. (14) showed that differences between routine methods can be explained to a large extent by faulty calibration. This is certainly also true in the present case. In the candidate reference method a molar absorption coefficient of 7550 m^2/mol (75500 l · mol^{-1} · cm^{-1}) (at 598 nm) is used, whereas the molar absorption coefficient for the routine method is 6620 m^2/mol (66200 l · mol^{-1} · cm^{-1}) (at 578 nm), which corresponds to 6940 m^2/mol (69400 l · mol^{-1} · cm^{-1}) at 598 nm.

These differences between the molar absorption coefficients would give rise to a difference of 8% between the analytical values; in the present comparison, however, the difference was only 3.9% (at 200 μmol/l). It therefore appears that albumin-bound bilirubin is not

Tab. 4. Statistical evaluation of the comparison of 5 routine methods with the reference method (N = number of samples, a = intersection of the axis, b = slope of the regression line, r = correlation coefficient).

No.	Method	N	a	b	r
2	*Jendrassik & Grof*	77	1.66	1.031**	0.997
3	DPD	89	12.57*	1.041**	0.997
4	*Hertz*	77	6.81*	1.015	0.997
5	*Vink*	77	5.05*	0.992	0.999
6	Bilimeter	58	17.31*	1.076**	0.991

* The hypothesis a = 0 is rejected ($p < 0.05$)
** The hypothesis b = 1 is rejected ($p < 0.05$)

completely converted into azobilirubin in the routine method. This is mainly due to the molar ratio of sulphanilic acid to sodium nitrite, which is 16:1 in *Doumas'* method and 6:1 in the routine method. The large excess of sulphanilic acid is needed, however, to ensure the maximal rate of the coupling reaction (15). On the other hand, if a ratio of 3:1 is used, the analytical values are about 4% lower (16).

The absence of EDTA could also be responsible for the differences to *Doumas'* method. *Holtz & van Dreumel* (17) reported that copper interfered with the production of the azo-pigment, by contributing to the formation of an azo-bilirubin-copper complex. This can be prevented by addition of EDTA to the reaction mixture (17).

According to our present findings, the accuracy of *Jendrassik & Grof's* method can be improved by standardization with sample P1, or by the use of a redeterminated molar absorption coefficient (6878 m^2/mol = 68780 $l \cdot mol^{-1} \cdot cm^{-1}$ at 578 nm).

The results from the 2,5-dichlorophenyldiazonium method (DPD method) are especially surprising. The regression analysis suggests the existence of errors arising from the calibration (the slope of the regression line is 1.041, which is significantly different from 1), and from the blank value (the intersection of the axis lies at 12.57 µmol/l, which is significantly different from zero).

The reasons for this are purely speculative, since the method is published only in abstract form, and there is no information on its specificity. The only reported interference is that by indican (18, 19). Data for the concentration of indican in neonatal sera are not available, but increased concentrations have been described in pregnancy (20), and it is conceivable that indican is able to cross the placenta. Redetermination of the molar absorption coefficient (4773 vs. 4585 m^2/mol corresponding to 47730 instead of 45850 $l \cdot mol^{-1} \cdot cm^{-1}$) did indeed improve the accuracy of the DPD method, but there still remains the problem of an "unspecific" contribution averaging 12.6 µmol/l.

When the calibration was performed with P1, the deviation from the reference values was much less in the range of interest for neonatal determinations (300 µmol/l: 1.1%, 200 µmol/l: 3.1%, 100 µmol/l: 9%); in the lower measurement range, however, calibration with P1 did not lead to satisfactory accuracy.

The method of *Hertz, Dybkaer & Lauritzen*, which is accorded the status of a "reference method" by many authors was regarded to be suitable not only for the determination of bilirubin in neonatal serum, but also in adult serum containing both unconjugated and conjugated bilirubin. The authors based their calculation formula on the analysis of 170 samples, which included only 31 neonatal sera (9); the procedure of *Jendrassik & Grof* was used as the "reference method." In the present comparison, however, *Hertz*'s method gave results that were not only significantly different from those of the reference method, but also from those of *Jendrassik & Grof's* method. The regression equation (y = *Hertz*'s method, x = *Jendrassik & Grof*'s method) was $y = 0.969x + 9.25$ ($b \neq 1$, $a \neq 0$, $p < 0.05$).

Values from *Hertz*'s method differed from those of the reference method by 10.8% at 100 µmol/l and 3.8% at 300 µmol/l. Similar differences were reported by *Vink* (10) and by *Blijenberg* (21). Since the protein matrix influences the results of *Hertz*'s method (5, 21, 22), the differences between *Hertz*'s method and the reference method were even greater when P1 was used for calibration (tab. 5).

The best level of agreement with the reference method was shown by *Vink*'s method (10), and this was not altered by calibration with P1. Similarly satisfactory results were reported by *Vink* (10) and by *Hajzer* (23).

The greatest deviation from the reference method values was shown by the bilimeter results. In addition to a slope of 1.076 (significantly different from unity), the regression equation showed an intersection of the axis at 17.3 µmol/l, which was significantly different from zero. This constant error can be explained by

Tab. 5. Statistical evaluation of the comparison of methods, using control serum P1 as the standard for calculation of the results.

No.	Method	N	a	b	r
2	*Jendrassik & Grof*	77	1.66	1.001	0.997
3	DPD	89	11.77*	0.972**	0.997
4	*Hertz*	77	6.95*	1.031**	0.997
5	*Vink*	77	4.57*	1.001	0.999
6	Bilimeter	58	17.84*	1.109**	0.991

* The hypothesis a = 0 is rejected ($p < 0.05$)
** The hypothesis b = 1 is rejected ($p < 0.05$)

the non-linear calibration curve of the apparatus (13). When the calibration was performed with P1 instead of "Sta-bil", this difference became even greater. As in *Hertz*'s method, an effect of the matrix on the results is also probably responsible for this difference. At the moment, an improvement of accuracy only appears possible by using a serum pool for the calibration. Since the calibration line is non-linear, a two-point calibration will give approximately correct results even when only in a limited measurement range (13).

Conclusion

The present study shows that differences between routine methods and the reference method observed in interlaboratory collaborative studies (2, 3) are also observed in the analysis of neonatal serum samples. For both chemical methods (*Jendrassik & Grof*'s and the DPD method), the magnitude of this difference is practically the same for control sera and patient sera.

The accuracy of *Jendrassik & Grof*'s method, and to a limited extent that of the DPD method, can be improved by recalculation of the molar absorption coefficient, or by using P1 for calibration. P1 ("candidate standard for use in calibration of total bilirubin in serum") is, however, unsuitable for calibration of *Hertz*'s direct spectrophotometric method or the bilimeter, because the inaccuracy of both is increased by the matrix of human albumin. This problem can be circumvented by replacing *Hertz*'s method with *Vink*'s method, which shows excellent agreement with the reference method. The bilimeter results can only be improved by calibrating the apparatus with a serum pool, whose analytical value has been established by the reference method (13).

References

1. Gemeinsame Empfehlung der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe, der Deutschen Gesellschaft für Kinderheilkunde und der Berufsverbände zur Frage der Phototherapie bei der Neugeborenen-Hyperbilirubinämie. Gynäkologie und Geburtshilfe (1983), *3/83*, 24–25.
2. Blijenberg, B. G., Brouwer, H. J. Roetering, H. A. & Leijnse, B. (1984) Surveys of neonatal bilirubin – an evaluation. J. Clin. Chem. Clin. Biochem. *22*, 609–612.
3. Röhle, G., Schlebusch, H., Geilenkeuser, W. J. & Kruse, R. (1988) External quality control in the determination of neonatal bilirubin – an approach to the improvement of results. J. Clin. Chem. Clin. Biochem. *26*, 441–446.
4. Doumas, B. T., Kwok-Cheung, P. P., Perry, B. W., Jendrzejczak, B., Mc Comb, R. B., Schaffer, R. & Hause, L. L. (1985) Candidate reference method for determination of total bilirubin in serum: Development and Validation. Clin. Chem. *31*, 1779–1789.
5. Vink, K. L. J., van Dreumel, R. J., Schuurman, W., Wikkeling, R. H., van Gansewinkel, R., Phielix, C. J. & Koedam, J. C. (1987) A candidate standard for use in calibration of total bilirubin in serum. Clin. Chem. *33*, 1817–1821.
6. Wikkeling, R. H., van Dreumel, R. J., Phielix, C. J. & Koedam, J. C. (1988) Primary bilirubin standards. Fresenius Z. Anal. Chem. *330*, 425–426.
7. Jendrassik, L. & Grof, P. (1938) Vereinfachte photometrische Methode zur Bestimmung des Blutbilirubins. Biochem. Z. *297*, 81–89.
8. Wahlefeld, A. W., Herz, G. & Bernt, E. (1972) Modification of the Malloy-Evelyn method for a simple determination of total bilirubin in serum. Scand. J. Clin. Lab. Invest. *29*, Suppl. 126, Abstract 11.12.
9. Hertz, H., Dybkaer, R. & Lauritzen, M. (1974) Direct spectrometric determination of the concentration of bilirubins in serum. Scand. J. Clin. Lab. Invest. *33*, 215–230.
10. Vink, K. L. J., Schuurman, W. & van Gansewinkel, R. (1988) Direct spectrophotometry of bilirubin in serum of the newborn, with use of caffeine reagent. Clin. Chem. *34*, 67–70.
11. Kupke, I. R. (1977) Die photometrische Mikrobestimmung von Bilirubin im unverdünnten Kapillarplasma von Neugeborenen. Z. Geburtsh. u. Perinat. *181*, 456–459.
12. Passing, H. & Bablok, W. (1983) A new biometrical procedure for testing the equality of measurements from two different analytical methods. J. Clin. Chem. Clin. Biochem. *21*, 709–720.
13. Schlebusch, H., Liappis, N., Röhle, G. & Schneider, Ch. (1989) Zur Kalibration von Bilirubinometern. Lab. Med. *13*, 364–368.

14. Doumas, B. T., Perry, B. W., Sasse, E. A. & Straumfjord, J. V. (1973) Standardization in bilirubin assays: Evaluation of selected methods and stability of bilirubin sources. Clin. Chem. *19*, 984–993.
15. Landis, J. B. & Pardue, H. L. (1978) Kinetics of the reactions of unconjugated and conjugated bilirubins with p-diazobenzenesulfonic acid. Clin. Chem. *24*, 1690–1699.
16. Lo, D. H. & Wu, T. W. (1983) Assessment of the fundamental accuracy of the Jendrassik-Grof total and direct bilirubin assays. Clin. Chem. *29*, 31–36.
17. Holtz, A. H. & van Dreumel, H. J. (1968) Stabilization of azobilirubin solution by EDTA. Clin. Chim. Acta *20*, 355–357.
18. Ertingshausen, G., Fabiny Byrd, D. L., Tiffany, T. O. & Casey, S. J. (1973) Single-reagent method for rapid determination of total bilirubin with the "Centrifichem" analyzer. Clin. Chem. *19*, 1366–1369.
19. Poow, R. & Hinberg, I. H. (1985) Indican interference with six commercial procedures for measuring total bilirubin. Clin. Chem. *31*, 92–94.
20. Henning, N. (1966) *Klinische Laboratoriumsdiagnostik*. 3. Auflage, Urban u. Schwarzenberg, München, pp. 138–139.
21. Blijenberg, B. G., Roetering, H. A. & Leijnse, B. (1987) Reflections on the standardization of total bilirubin in neonatal serum. J. Clin. Chem. Clin. Biochem. *25*, 177–181.
22. Vink, K. L. J., Schuurman, W. & van Gansewinkel, R. (1986) Use of the caffeine reagent in direct spectrophotometry of bilirubin. Clin. Chem. *32*, 1389–1393.
23. Hajzer, S. (1989) Comparison of direct spectrophotometric determinations of bilirubin with candidate reference method in sera of newborns. J. Clin. Chem. Clin. Biochem. *27*, 445–449.

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